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Project Summary

Bioremediation of BTEX, Naphthalene, and Phenanthrene in Aquifer Material Using Mixed Oxygen/Nitrate Electron Acceptor Conditions

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The goal of the research described herein was to examine the feasibility of biodegradation of mono and polycyclic aromatic hydrocarbons typically present in a manufactured gas processing (MGP) site groundwater and subsurface sediments under mixed oxygen/denitrifying conditions. The principal hypothesis considered in this research is that biodegradation of certain mono and polycyclic aromatic hydrocarbons occurs under mixed oxygen/denitrifying conditions and that the rate and extent of biodegradation is greater under these conditions than traditional single electron acceptor schemes. To test this hypothesis, laboratory experiments were designed to compare biodegradation under mixed electron acceptor conditions with biodegradation under single electron acceptor schemes.

This Project Summary was developed by EPA's National Risk Management Research Laboratory's Subsurface Protection and Remediation Division, Ada, OK, to announce key findings of the research project that is fully documented in a separate report of the same title (see Project Report ordering information at back).

Introduction

This research project included three phases: (1) screening of site aquifer material for microorganisms which can successfully biodegrade model aromatic compounds under aerobic and denitrifying conditions (facultative anaerobes); (2) batch studies to assess the biodegradation of the model compounds under mixed oxygen/nitrate electron acceptor conditions compared with biodegradation under aerobic and anaerobic denitrifying conditions; and (3) aquifer material column studies to confirm the findings of the batch studies and to better simulate mixed oxygen/denitrifying remediation in the subsurface. Specific experiments included in each phase of the research are summarized in Table 1.

Methods and Results

Phase I - Microbial Characterization and Assessment

For in situ biodegradation with mixtures of oxygen and nitrate to be successful, bacteria that are capable of using both oxygen and nitrate as electron acceptors must be present at the remediation site. A survey of the MGP site sediments demonstrated that viable bacteria were present at the site and at a variety of depths and locations. Some of these bacteria could be cultured under aerobic and anaerobic conditions suggesting that facultative anaerobic bacteria are present at the site. Mineralization assay results demonstrated that indigenous site bacteria were capable of aerobic biodegradation of a number of compounds including benzene, toluene, naphthalene and phenanthrene and anaerobic mineralization of naphthalene. The extent of substrate mineralization under aerobic conditions ranged from 0 to 91%. The extent of naphthalene mineralization after 80 days of incubation under denitrifying conditions was as high as 16%. Mineralization assays conducted for 30 days using liquid enrichments of these aquifer bacteria (no aquifer solids were present)

Table 1. *Summary of the Experiments Conducted in Each of the Three Research Phases*

PHASE I - MICROBIAL ASSESSMENT		
Sample Collection	*Batch Study 1* *Single substrate, aquifer material microcosms*	*Batch Study 2* *Single substrate, liquid enrichment microcosms*
PHASE II - BATCH STUDIES OF MIXED OXYGEN/NITRATE ELECTRON ACCEPTOR CONDITIONS		
Batch Study 1 *Mixed substrates, liquid enrichment microcosms*	*Batch Study 2* *Mixed substrates, aquifer material microcosms*	*Batch Study 3* *Mixed substrates, aquifer material microcosms, electron acceptor replaced over time*
PHASE III - COLUMN STUDIES OF MIXED OXYGEN/NITRATE ELECTRON ACCEPTOR CONDITIONS		
Column A *Anaerobic/microaerophilic single-port injection*	*Column B* *Anaerobic/microaerophilic single-port injection*	*Column C* *Aerobic single-port and multi-port injection*

under anaerobic denitrifying conditions did not yield mineralization of the target compounds. The biodegradation rates were so slow that 30 days was not long enough to observe mineralization of the target substrates. Notwithstanding the lack of mineralization of the model compounds in the liquid enrichment microcosms, sufficient evidence of denitrifying activity was observed in the culture fluids (conversion of nitrate to nitrite).

Phase II - Batch Studies Under Mixed Electron Acceptor Conditions

Batch Study 1 - Mixed Substrates - Enrichment Microcosms - Mixed Electron Acceptors. Batch microcosm experiments proved to be a successful method to screen for biodegradation of BTEX, naphthalene and phenanthrene under varying combinations of oxygen and nitrate. The aromatic compounds biodegraded under varying combinations of oxygen and nitrate are summarized in Table 2.

Biodegradation was defined as 10% loss of substrate relative to controls. With the exception of toluene, oxygen appeared to be the key to removal of the aromatic hydrocarbons. Increased levels of oxygen yielded an improvement in the extent of compound removal in the batch microcosms. Despite the improved biodegradation with increasing oxygen concentration, the denitrifying enrichment was sensitive to extremely high levels of oxygen (30 mg O_2/L). Although the bacteria were able to use oxygen under these conditions, a lag time occurred before significant biodegradation was observed. No lag time was observed during biodegradation when air saturated conditions were provided (7.6 mg O_2/L) resulting in good removal of all compounds (except benzene in some microcosms). Providing oxygen in excess of the stoichiometric requirements for aerobic biodegradation did not necessarily yield a greater extent of biodegradation of aromatic compounds. It appears that the rate of biodegradation of all compounds may be enhanced by providing a lower level of oxygen (i.e., 7 to 8 mg O_2/L) which may be less toxic to facultative anaerobic bacteria.

Under microaerophilic conditions, the enrichment was able to use oxygen to degrade naphthalene without any lag time suggesting that the enzymes for aerobic biodegradation (oxygenases) are easily induced under conditions where the oxygen concentration is equivalent to or greater than 1.5 mg/L. At levels of oxygen less than 1 mg/L, only toluene biodegradation was observed. Toluene removal was not initiated until the oxygen was nearly depleted. In this case, the oxygen removal observed prior to toluene biodegradation may have been due in part to some oxygen removal or detoxifying mechanism or to endogenous respiration, and appeared to be required before significant anaerobic denitrification was observed. Results of experiments with toluene as the sole substrate suggested that in the absence of competing, aromatic hydrocarbon substrates (i.e., benzene, ethylbenzene, *m*-xylene, naphthalene and phenanthrene), oxygen may act as an electron acceptor during biodegradation of

Table 2. *Aromatic Hydrocarbons Degraded Under Various Combinations of Oxygen and Nitrate in Batch Liquid Enrichment Microcosms*

Nitrate (mg/L)	*Oxygen (mg/L)*						
	0	*0.5*	*1.0*	*1.5*	*2.0*	*7.0*	*30.0*
10	**T**	**T**	**n.t.**	**T,N**	**T,N**	**B,T,E,*m*-X,N,P**	**B,T,E,*m*-X,N,P**
50	**T**	**T**	**T,E**	**T,N**	**T,N**	**B,T,E,*m*-X,N,P**	**n.t.**
150	**T**	**T**	**T,E**	**T,N**	**T,N**	**B,T,E,*m*-X,N,P**	**n.t.**
400	**T**	**T**	**T,E**	**T,N**	**T,N**	**B,T,E,*m*-X,N,P**	**n.t.**

B *= benzene,* ***T*** *= toluene,* ***E*** *= ethylbenzene,* ***m-X*** *= m-xylene,* ***N*** *= naphthalene and* ***P*** *= phenanthrene,* ***n.t.*** *= not tested*

toluene. The rate of oxygen use was slow at high initial levels of oxygen and faster at microaerophilic levels. Zero order rates for oxygen consumption ranged from 0.016-0.032 mg/L-hour under microaerophilic conditions and was as slow as 0.0066 mg/L-hour under oxygen saturation conditions (O_2 ~ 30 mg/L).

Batch Study 2 - Mixed Substrates - Aquifer Material Microcosms - Mixed Electron Acceptors. The objective of Batch Study II was to assess the impact of sediments on the transformation of a mixture of aromatic hydrocarbons (BTEX, naphthalene, and phenanthrene) under mixed electron acceptor conditions. The results of the batch microcosm experiments using sediment as inocula under aerobic, microaerophilic and denitrifying conditions were not distinguishable. Only toluene was degraded and mineralization of toluene occurred under denitrifying conditions. Residual oxygen was consumed in the microcosms within 24 hours of experimental setup. Oxygen consumption was presumably due to significant abiotic oxygen demands associated with sediments cored from anaerobic regions of the source aquifer. Additional oxygen demands may be attributable to the degradation of labile organic carbon associated with the sediments. These results reveal that abiotic oxygen demands must be accounted for when batch experiments are conducted to estimate kinetic parameters for the design of in situ bioremediation processes.

Batch Study 3 - Mixed Substrates - Aquifer Material Microcosms - Electron Acceptor Replenished Over Time. The primary goals of Batch Study 3 were to: 1) satisfy the abiotic demand for oxygen in the microcosms to allow for study of the biotic oxygen demand of biodegradation, and 2) quantify the level of oxygen at which aerobic degradation of mixed aromatic substrates (BTEX and naphthalene) was inhibited and denitrification was initiated in batch sediment microcosms. The results of this set of experiments revealed that both oxygen and nitrate were utilized as terminal electron acceptors under microaerophilic conditions (O_2 concentration < 2 mg/L). Concurrent use of oxygen and nitrate as terminal electron acceptors occurred when aqueous oxygen concentrations were below 2.0 mg/L. Toluene was degraded under denitrifying conditions while benzene, ethylbenzene, *m*-xylene and naphthalene were degraded using oxygen as the electron acceptor. Denitrifying activity and toluene transformation were observed in the presence of slightly higher bulk solution dissolved oxygen concentrations than observed in the liquid enrichment microcosms (Batch Study 1). The sediments likely exerted additional oxygen demand such that additional oxygen was required to achieve the same results as were observed in Batch Study 1. The presence of sediments may have resulted in microsite dissolved oxygen concentrations below that of the bulk solution.

Naphthalene, *m*-xylene and toluene were preferentially degraded to a greater extent and at a faster rate than benzene and ethylbenzene. Significant benzene and ethylbenzene biotransformation did not typically occur until toluene, naphthalene, and *m*-xylene were removed from the microcosms (Figure 1).

A zero-order rate model (independent of substrate concentration) provided the best fit to the experimental data. The rate of substrate transformation was significantly greater under aerobic conditions than microaerophilic conditions. The rates of transformation for each of the substrates were relatively constant under microaerophilic conditions for dissolved oxygen concentrations ranging from

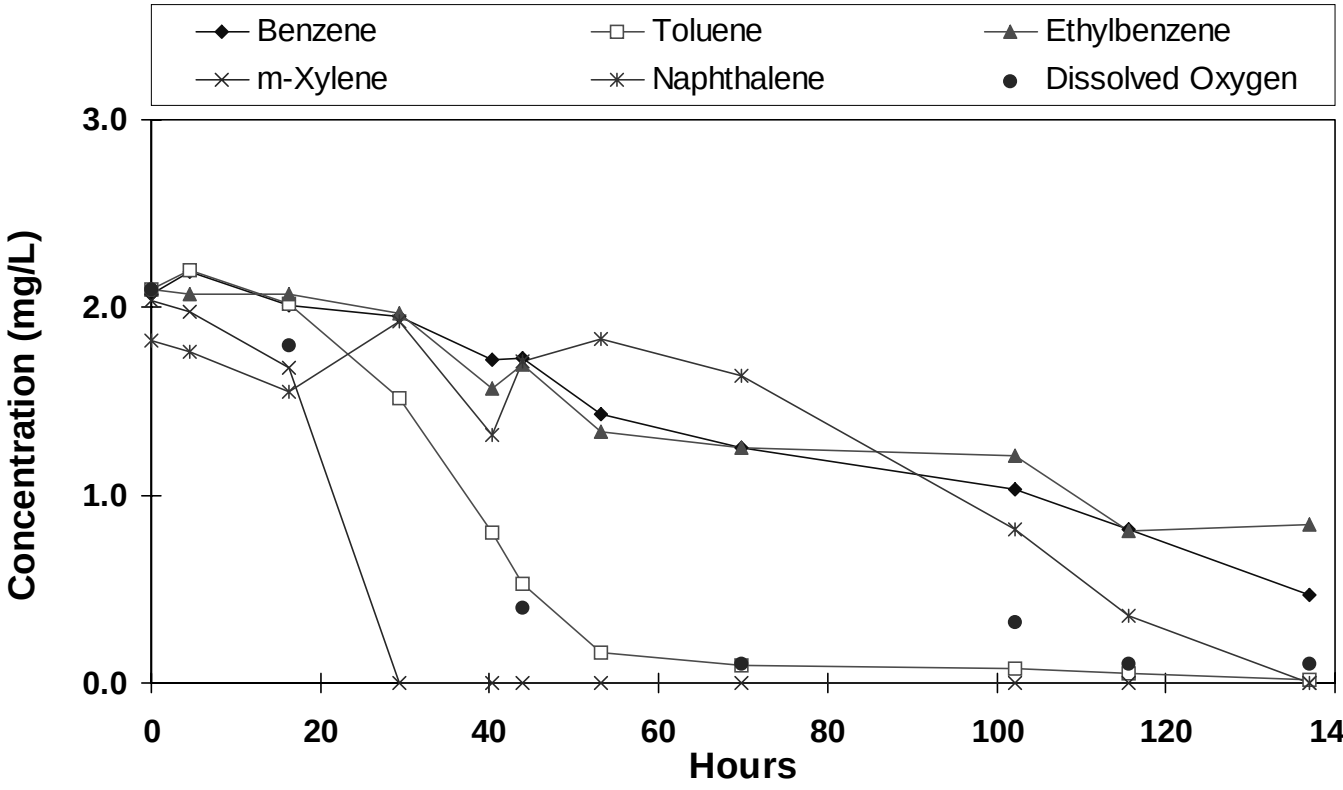

Figure 1 *- Substrates and dissolved oxygen remaining in Sediment Microcosm #1 with an initial oxygen concentration of 2 mg/L.*

0.45 mg/L-hour to 1.1 mg/L-hour. Oxygen concentration controlled biodegradation of this suite of aromatic hydrocarbons in batch sediment microcosms. Oxygen levels also controlled denitrification as well as the rate and extent of substrate removal. Providing a mixture of microaerophilic and denitrifying conditions did not necessarily improve biodegradation when compared with oxygen alone as long as oxygen was maintained between 0.45 and 1.1 mg/L. Denitrification appears to play a role in substrate removal only when the supply of oxygen is limited and finite.

Phase III - Simulation of In Situ Treatment in Soil Columns Under Mixed Electron Acceptor Conditions

The overall objective of this research was to evaluate the biodegradability of a mixture of aromatic compounds using mixed oxygen/nitrate electron acceptors under conditions which simulate a contaminated groundwater aquifer. This was accomplished using saturated sediment columns. Biodegradation of BTEX and naphthalene was evaluated under the following electron acceptor conditions:

Microaerophilic - 2 mg/L O_2 and 150 mg/L NO_3
Microaerophilic - 1 mg/L O_2 and 150 mg/L NO_3
Aerobic - 8.6 mg/L O_2 and 55 mg/L NO_3

As with the batch microcosm studies, the most successful biodegradation of the mixture of aromatic hydrocarbons occurred under aerobic conditions (~ 8.6 mg O_2/L) in the presence of nitrate. Excellent toluene removal was also achieved in all columns with all levels of oxygen (i.e., 0, 1, 2 and 8.6 mg O_2/L) except in the absence of nitrate underscoring the importance of nitrate to toluene remediation in these sediments. By increasing the concentration of oxygen, the number of compounds and the extent of their biodegradation was enhanced. Benzene, ethylbenzene, *m*-xylene and naphthalene were recalcitrant in the absence of oxygen. Providing microaerophilic levels of oxygen ($\leq$ 2 mg O_2/L) enhanced the removal of ethylbenzene, *m*-xylene and naphthalene but fully aerobic conditions (> 7 mg O_2/L) allowed for some removal of all compounds with naphthalene and toluene completely transformed (> 95%).

The extent of naphthalene removal was a function of oxygen concentration and increased with an increase in oxygen concentration. The proportion of naphthalene that was converted to carbon dioxide and intermediates was unaffected by oxygen concentration. Therefore, oxygen concentration probably controls the initial step(s) in naphthalene breakdown and may not be involved in the mineralization of the resulting intermediates or the decay of microbial cells. Naphthalene removal was observed in the column which received 2 mg O_2/L but not in the column which received 1 mg O_2/L. These results support the findings of batch liquid enrichment microcosm studies which concluded that there was a threshold oxygen concentration (1.5 mg O_2/L) below which naphthalene removal did not occur. However, for batch electron acceptor replenishment studies (Phase II, Batch Study 3), naphthalene was transformed at aqueous oxygen concentrations less than 0.5 mg/L.

Toluene and naphthalene removal ceased once nitrate was removed from the microaerophilic columns. When nitrate was restored to the column influent, toluene and naphthalene removal continued (with 2 mg O_2/L) providing further evidence that nitrate is required for biodegradation of toluene and naphthalene in these aquifer sediments. The role of O_2 and NO_3 in the removal of toluene and naphthalene in a microaerophilic sediment column is illustrated in Figure 2. Nitrate consumption and nitrite production increased in the aerobic column in response to an increase in the influent toluene concentration. Furthermore, sampling along the length of the aerobic column revealed that the extent of toluene transformation could be correlated to the consumption of nitrate and the production of nitrite along the length of the column in the presence of aqueous pore oxygen concentrations greater than 2 mg/L. Substantial denitrifying activity was observed in the aerobic column in the presence of pore dissolved oxygen concentrations as high as 5 mg/L. Either aerobic levels of oxygen did not inhibit denitrifying activity, or denitrifying activity occurred in microsites or within a biofilm where dissolved oxygen concentrations may have been lower than in the bulk pore space. These data support the belief that nitrate may enhance mineralization by acting as an alternative electron acceptor or simply by stimulating additional cell formation. Regardless of its role, aerobic bioremediation is enhanced by the addition of nitrate in aquifer sediments harboring denitrifying bacteria.

Conclusions

The results of these experiments have important implications for in situ bioremediation. Providing some level of oxygen resulted in better substrate removal than anaerobic denitrifying conditions except in the case of toluene where oxygen did not provide any benefit in terms of the extent of toluene removal. There were no benefits to providing microaerophilic levels of oxygen ($\leq$ 2 mg/L) in combination with nitrate when compared with higher levels of oxygen (7 and 30 mg O_2/L). Moderate, yet aerobic levels of oxygen in combination with nitrate rather than high concentrations (30 mg O_2/L) resulted in comparable substrate removal and faster kinetics.

Providing low levels of oxygen in combination with nitrate during in situ bioremediation rather than only high levels of oxygen may accomplish or yield the following benefits: 1) low levels of oxygen are not toxic to denitrifying bacteria allowing for facultative use of both oxygen and nitrate as electron acceptors; 2) low levels of oxygen are less expensive to maintain in the subsurface; and 3) it is easier to maintain a low residual oxygen concentration in the subsurface than a high concentration due to the many oxygen demands/sinks. An in situ bioremediation scheme which combines moderate aerobic (7 mg/L O_2) and denitrifying conditions will likely prove more successful than solely aerobic remediation for the long term remediation of aromatic hydrocarbons.

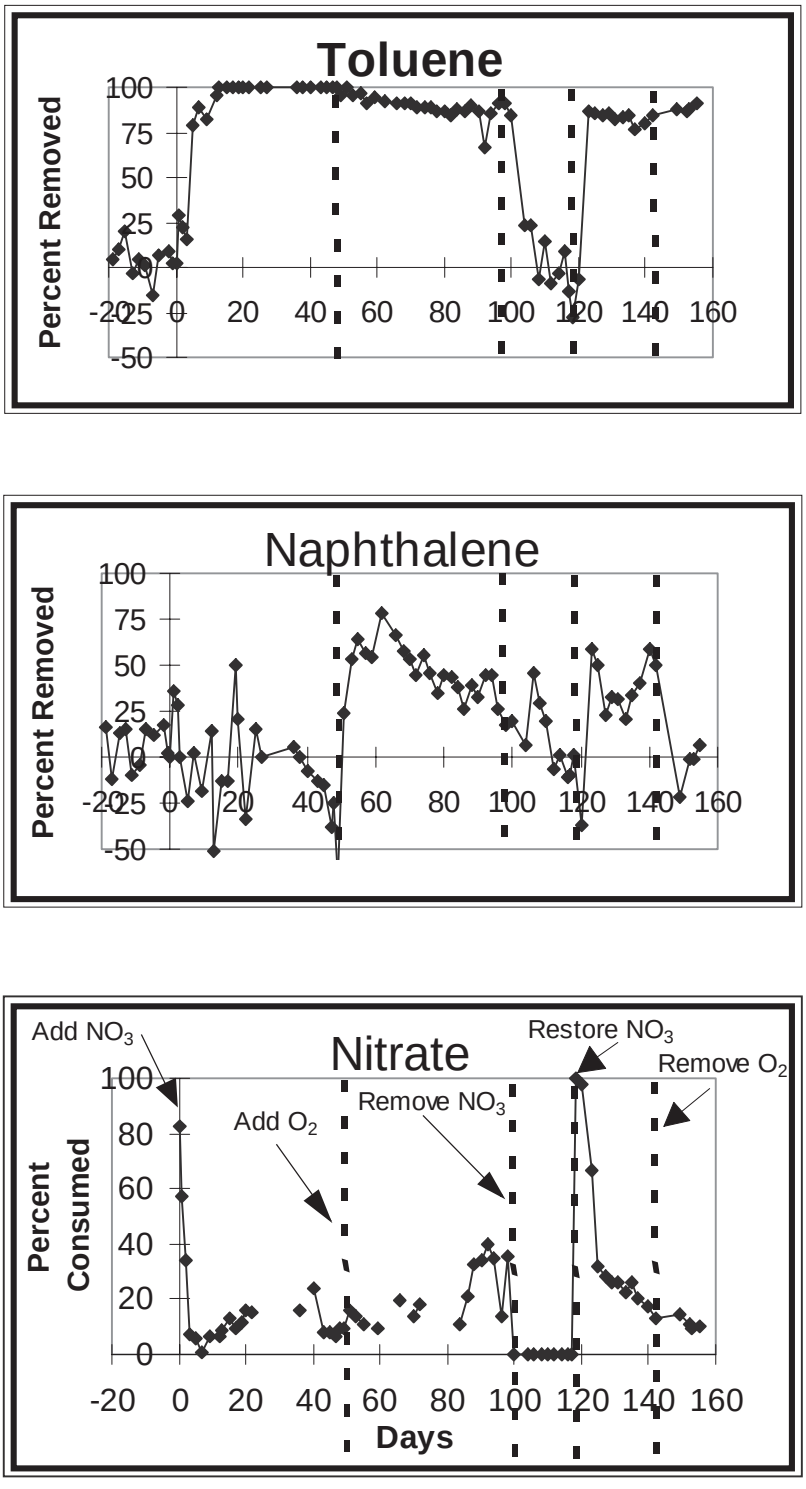

Figure 2. *Percent toluene and naphthalene removed and nitrate consumed in Column A over time under anaerobic, mixed oxygen/ nitrate and aerobic conditions (2 mg/L oxygen and 150 mg/L nitrate).*

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The complete report, entitled "Bioremediation of BTEX, Naphthalene, and Phenanthrene in Aquifer Material Using Mixed Oxygen/Nitrate Electron Acceptor Conditions," (Order No. PB95-X; Cost: $X.00, subject to change) will be available only from:

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